European Food Safety Authority

The EFSA Journal (2009) 1306, 1-23

SCIENTIFIC OPINION

Marine biotoxins in shellfish – Summary on regulated marine biotoxins[1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question No EFSA-Q-2009-00685)

Adopted on 13 August 2009

PANEL MEMBERS

Jan Alexander, Diane Benford, Alan Boobis, Sandra Ceccatelli, Jean-Pierre Cravedi, Alessandro Di Domenico, Daniel Doerge, Eugenia Dogliotti, Lutz Edler, Peter Farmer, Metka Filipič, Johanna Fink-Gremmels, Peter Fürst, Thierry Guerin, Helle Katrine Knutsen, Miroslav Machala, Antonio Mutti, Josef Schlatter, Rolaf van Leeuwen and Philippe Verger

SUMMARY

The European Commission (EC) has requested the European Food Safety Authority (EFSA) to summarise the outcome of the adopted opinions on marine biotoxins that are currently regulated in European Union (EU) legislation, namely the opinions on okadaic acid (OA) and analogues, azaspiracid (AZA)-group toxins, yessotoxin (YTX)-group toxins, saxitoxin (STX)-group toxins, pectenotoxin (PTX)-group toxins and domoic acid (DA). The EC asked EFSA to address the current EU limits with regard to human health and methods of analysis.

This opinion provides an overview of the EU regulatory limits, the acute reference doses (ARfD) set by EFSA, the exposure levels resulting from consumption of shellfish on the EU market, the available methods of analysis, certified calibrants and reference materials, the influence of processing on the levels of the toxins and the relative potency of the analogues of the six regulated marine biotoxins.

Based on the established ARfDs it is concluded that the current EU regulatory limit values for OA-, AZA-, STX-group toxins and DA are not sufficiently protective for high consumers. For YTX- and PTX-group toxins, the EU limit values are sufficiently protective.

The mouse bioassay (MBA) is the official reference method for lipophilic biotoxins. The Panel on Contaminants in the Food Chain (CONTAM Panel) noted that this bioassay has shortcomings and is not considered an appropriate tool for control purposes because of the high variability in results, the insufficient detection capability and the limited specificity.

[1] For citation purposes: Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. *The EFSA Journal* (2009) 1306, 1-23

© European Food Safety Authority, 2009

Recently developed alternatives to the reference methods for the determination of the marine biotoxins with lower limits of detection (LOD) have successfully been tested in prevalidation studies. Method performance criteria should be stipulated where possible and validation by interlaboratory trials should be the long-term objective.

Key words: **Marine biotoxin, okadaic acid, dinophysis toxin, azaspiracid, yessotoxin, saxitoxin, pectenotoxin, domoic acid, shellfish, bivalve mollusc, mouse bioassay (MBA), acute reference dose, portion size, method of analysis, human health, risk assessment, summary.**

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin[2] establishes maximum levels for marine biotoxins in live bivalve molluscs.

Commission Regulation (EC) No 2074/2005[3] of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004[4] of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004[5] of the European Parliament and of the Council and Regulation (EC) No 882/2004[6] of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004[7] of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 establishes the recognised testing methods for detecting marine biotoxins.

In July 2006 the Commission requested EFSA to provide a scientific opinion to assess the current EU limits with regard to human health and methods of analysis for various marine biotoxins as established in the EU legislation, including new emerging toxins.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is requested to provide an opinion summarising the outcome of the adopted opinions on marine biotoxins, namely okadaic acid, azaspiracid, yessotoxin, saxitoxin, pectenotoxin and domoic acid, addressing the current EU limits with regard to human health and methods of analysis.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on Marine Biotoxins for the presentation of this opinion: Jan Alexander, Diane Benford, Luis Botana, Peter Fürst, Gerhard Heinemeyer, Philipp Hess, Angelika Preiss-Weigert, Gian Paolo Rossini, Hans van Egmond, Rolaf van Leeuwen and Philippe Verger.

2 OJ L226, 25.6. 2004, p. 22-82
3 OJ L338, 22.12. 2005, p. 27-59
4 OJ L 139, 30.4.2004, p. 55–205
5 OJ L 139, 30.4.2004, p. 206–320
6 OJ L 165, 30.4.2004, p. 1–141
7 OJ L 139, 30.4.2004, p. 1–54

ASSESSMENT

1. Introduction

The European Commission (EC) asked the European Food Safety Authority (EFSA) to summarise the outcome of the adopted opinions on marine biotoxins that are currently regulated in the European Union (EU) legislation, namely the opinions on okadaic acid (OA) and analogues[8], azaspiracid (AZA)-group toxins[9], yessotoxin (YTX)-group toxins[10], saxitoxin (STX)-group toxins[11], pectenotoxin (PTX)-group toxins[12] and domoic acid (DA)[13]. The EC asked EFSA to address the current EU limits with regard to human health and methods of analysis.

The Panel on Contaminants in the Food Chain (CONTAM Panel) reports in the current opinion the EU limit values and the exposure due to the consumption of a large portion at the EU regulatory limit, as calculated in the opinions. Additionally it summarises the acute reference doses[14] (ARfDs) derived for the evaluated marine biotoxins reported in the respective opinions and the corresponding concentration of marine biotoxins per kg of shellfish meat that would not lead to exceedance of the ARfD when consuming a large portion (400 g) of shellfish. Regarding the methods of analysis, the current opinion reports the reference methods prescribed in the EU legislation, alternatives to these methods used for the determination of the different marine biotoxins, including their ability to perform at the regulatory limits, their limits of detection (LOD) and limits of quantification (LOQ) and information on their specificity. In addition, information on interlaboratory validation and standardisation and on the available certified calibrants and reference materials is presented. This opinion also includes a summary of the influence of processing (cooking, steaming, autoclaving) on the levels of the different marine biotoxins. Finally, it reports the toxicity equivalency factors (TEFs) adopted by the CONTAM Panel for regulated marine biotoxins.

More detailed information can be found in the opinions of the CONTAM Panel cited above and in the statement of the CONTAM Panel on the influence of processing on the levels of lipophilic marine biotoxins in bivalve molluscs[15].

2. Current EU regulatory limit values and the ARfDs set by EFSA

In view of the acute toxicity of the marine biotoxins, the CONTAM Panel decided to establish an ARfD for each of the toxin groups. It was not possible to establish longer term reference values, because there was a general lack of long term toxicity data. The CONTAM Panel compared these

8 The EFSA Journal (2008), 589, 1-62.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_589_okadaic_acid_en.pdf?ssbinary=true>
9 The EFSA Journal (2008), 723, 1-52.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_723_AZA_en,0.pdf?ssbinary=true>
10 The EFSA Journal (2008), 907, 1-62.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej_907_yessotoxin_en.pdf?ssbinary=true>
11 The EFSA Journal (2009), 1019, 1-76.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1019_saxitoxin_marine_biotoxins.pdf?ssbinary=true>
12 The EFSA Journal (2009), 1109, 1-47.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1109_pectenotoxins_en.pdf?ssbinary=true>
13 The EFSA Journal (2009), XXX, 1-XX.
14 The acute reference dose is the estimate of the amount of substance in food, normally expressed on a bodyweight basis (mg/kg or µg/kg of body weight), that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer on the basis of all known facts at the time of evaluation (JMPR, 2002).
15 The EFSA Journal (2009) 1016, 1-10

ARfDs to the exposure to marine biotoxins resulting from consumption of a single portion of shellfish rather than to average long-term exposure. In order to protect the high consumer against the acute effects of the marine biotoxins, the CONTAM Panel identified 400 g of shellfish meat as a realistic estimate of a large portion size, from the range of 95th percentiles for consumers of shellfish reported by five member states.

The CONTAM Panel calculated the exposure when consuming the large portion of shellfish containing marine biotoxins at the EU limit value and at the 95th percentile of the concentrations in samples that were compliant with the current EU regulation and that therefore could reach the EU market (Table 1). For OA-group toxins, but not for the other toxins, the exposure (96 µg OA equivalents/person) from eating a 400 g portion at the 95th percentile of the concentrations in samples currently on the EU market exceeds the exposure (64 µg OA equivalents/person) from eating a single 400 g portion at the EU limit value. This is because of the false negative results sometimes obtained when using the mouse bioassay (MBA) for official control of OA-group toxins at the current EU limit.

For OA- and AZA-group toxins the dietary exposures corresponding to the consumption of a single 400 g portion of shellfish meat containing the toxins at the current EU limit values are respectively 3-fold and 5-fold higher than the ARfDs. Such exposures could exert gastrointestinal effects in susceptible consumers. For DA and STX-group toxins, these exposures are respectively 4-fold and 10-fold higher than the ARfDs and are considered a concern for human health due to possible neurotoxic effects. Hence, the current EU regulatory limit values for the above mentioned toxins could result in exposures considered of human health concern, and are thus not sufficiently protective.

For YTX- and PTX-group toxins, the dietary exposures corresponding to the EU limit values are below and slightly above the ARfDs, and are considered not to pose any health risk.

The CONTAM Panel calculated the maximum concentrations (B) of marine biotoxins in shellfish meat that would ensure that the ARfD would not be exceeded when consuming a single 400 g portion of shellfish meat (Table 1). The ratio between the current EU limits (A) in shellfish meat and the concentration deduced from these ARfDs is given as B/A in the last column of Table 1. Except for YTX-group toxins these concentrations are below the current EU regulatory limit values for all marine biotoxins, indicating again that the current EU limit values are not sufficiently protective.

Table 1. Current EU limits, the exposure levels resulting from consumption of shellfish on the EU market, the ARfDs set by EFSA, and the corresponding concentrations in shellfish meat.

Toxin group	Current EU limits in shellfish meat (A)	Exposure by eating a 400 g portion at the EU limit [c]	Exposure from eating a 400 g portion at the 95th percentile of the concentrations in samples currently on the EU market	ARfD	Corresponding dose for a 60 kg adult	Maximum concentration in shellfish meat to avoid exceeding the ARfD, when eating a 400g portion (B)	Ratio B /A
OA and analogues	160 µg OA eq./kg SM[a]	64 µg OA eq./person (1 µg OA eq./kg b.w.)	96 µg OA eq./person (1.6 µg OA eq./kg b.w).	0.3 µg OA eq./kg b.w.	18 µg OA eq./person	45 µg OA eq./kg SM	0.28
AZA	160 µg AZA eq.[c]/kg SM	64 µg AZA1 eq./person (1 µg AZA1 eq./kg b.w.)	16 µg AZA1 eq./person (0.3 µg AZA1 eq./kg b.w.)	0.2 µg AZA1 eq./kg b.w	12 µg AZA1 eq./person	30 µg AZA1 eq./kg SM	0.19
PTX	160 µg OA eq./kg SM[a]	64 µg PTX2/person (1 µg PTX2 eq./kg b.w.)	32 µg PTX2/person (0.5 µg PTX2 eq./kg b.w.)	0.8 µg PTX2 eq./kg b.w	48 µg PTX2 eq./person	120 µg PTX2 eq./kg SM	0.75
YTX	1 mg YTX eq./kg SM	400 µg YTX eq./person (6.7 µg YTX eq./kg b.w.)	320 µg YTX eq./person (IT) (5.3 µg YTX eq./kg b.w.) 125 µg YTX eq./person (NO) (2.1 µg YTX eq./kg b.w.)	25 µg YTX eq./kg b.w	1500 µg YTX eq./person	3.75 mg YTX eq./kg SM	3.75
STX	800 µg PSP/kg SM[b]	320 µg STX eq./person (5.3 µg STX eq./kg b.w.)	< 260 µg STX eq./person (< 4.3 µg STX eq./kg b.w.)	0.5 µg STX eq./kg b.w	30 µg STX eq./person	75 µg STX eq./kg SM	0.09
DA	20 mg DA/kg SM	8 mg DA[d]/person (130 µg DA/kg b.w)	1 mg DA[d]/person (17 µg DA/kg b.w)	30 µg DA[d]/kg b.w	1.8 mg DA[d]/person	4.5 mg DA[d]/kg SM	0.23

SM: shellfish meat; eq.: equivalents; b.w.: body weight; ARfD: acute reference dose; PSP: paralytic shellfish poison; EU: European Union; IT: Italy; NO: Norway; OA: okadaic acid; PTX: pectenotoxin; YTX: yessotoxin; STX: saxitoxin; DA: domoic acid.

(a): For OA, dinophysistoxins and PTX, current regulation specifies a combination; however the CONTAM Panel concluded that PTX should be considered separately.

(b): In the Commission Regulation (EC) No 853/2004 a limit value of 800 µg PSP/kg SM is given. In the EFSA opinion, the CONTAM Panel adopted this figure as being expressed as µg STX equivalents/kg SM.

(c): The CONTAM Panel assumed that AZA equivalent should refer to AZA1 equivalents.

(d): Applies to the sum of DA and epi-DA.

When possible, the CONTAM Panel also compared the ARfD with the probabilistic dietary exposure estimated from the distributions of current consumption and occurrence data provided by the member states and Norway. The results are summarised in Table 2. The probabilities of exceeding the ARfD when consuming any portion of shellfish currently available on the European market were 20 % for OA-group toxins, 4 % for AZA-group toxins, about 1 % for DA, 0.2 % for PTX-group toxins, and <0.2 % for YTX-group toxins. For STX-group toxins, the CONTAM Panel could not comment on the risks associated with the consumption of shellfish currently reaching the market as it was not possible to make reliable estimates of the dietary exposure. This was due to the high portion of samples reported without a numerical value (below LOD), and the large impact of choosing either a lower bound or upper bound approach.

Finally, the CONTAM Panel calculated the percentage of samples compliant with the EU limit value but exceeding the concentration of marine biotoxins in shellfish meat resulting in exposure at the ARfD when consuming a single 400 g portion of shellfish. For AZA-, PTX- and YTX-group toxins and for DA this is less than 10 %, indicating that revising the regulatory limits would have a minor impact on the amount of product needing to be withheld from the market. For OA- and STX-group toxins more than 25 % of the samples exceed the concentration compatible with the ARfD. The figures shown are based on the lower bound approach; however the considerable uncertainty introduced by using a lower-or upper-bound approach,demonstrates the need for improved LODs for these toxin groups.

In addition, the CONTAM Panel noted that in the absence of formal reporting systems for human illness associated with exposure to marine biotoxins, it cannot be assumed that such illness does not occur under the current regulatory controls.

Table 2. Comparison of the ARfDs with the dietary exposures estimated from the distributions of the current consumption and occurrence data provided by the member states and Norway and the % of samples exceeding the concentrations compatible with the ARfD

Toxin group	Probability of exceeding the ARfD when consuming any single portion of shellfish on the EU market [a]	% of samples compliant with the EU limit but exceeding the concentration compatible with ARfD [b]
OA and analogues	20 %	32 % [c]
AZA	4 %	8.5 %
PTX	0.2 %	0.3 %
YTX	<0.2 %	-
STX	Exceedance of ARfD occurs but exposure could not be reliably estimated	25 %[c]
DA	1 %	3.5 %

EU: European Union; LB: lower bound; UB: upper bound; ARfD: acute reference dose
(a): probabilistic estimate based on the distributions of both occurrence and consumption
(b): The concentration was based on the 400 g portion size.
(c): based on lower bound estimate

3. Methods of analysis

3.1. Available methods of analysis

3.1.1. Reference methods

The reference methods prescribed in the EU regulation, including their ability to perform at the regulatory limits or their LODs and LOQs, information on their specificity and information on interlaboratory validation and standardisation are presented in Tables 3a and 3b. The major points of the methods are highlighted below.

3.1.1.1. Mouse bioassays (MBA) for lipophilic and STX-group toxins

The MBA, the officially prescribed method for the detection of OA-, AZA-, YTX-, PTX- and STX-group toxins, has two main protocols, one for lipophilic toxins (OA-, AZA- YTX- and PTX-group toxins), and one for STX-group toxins.

MBA for lipophilic biotoxins

- The ability of the MBA to detect OA-group toxins at the current EU regulatory limit value is inadequate, leading to false negative results in official controls.
- YTX-group toxins at concentrations below the regulatory limit value may cause positive results in the MBA protocol used for OA-group toxins.

- Other non-regulated bioactive compounds (e.g. spirolides, gymnodimines, fatty acids etc.) have also been reported to cause positive results in the MBA.
- The MBA is not capable of detecting concentrations of OA-, AZA- and PTX-group toxins below their current EU regulatory limit values.
- Since publication of the EFSA opinions on AZA-group toxins, information has become available on the ability of the MBA for lipophilic toxins to detect AZA-group toxins (Hess *et al.*, 2009). The paper reports that the MBA, in its harmonised form, is able to detect AZA-group toxins at the current EU regulatory limit of 160 µg/kg shellfish meat with a probability of 95 %. This appears adequate for implementation of the current official limit for AZA-group toxins. The authors also note that, due to the steepness of the dose-response curve, the assay only has a detection probability of ca. 5 % at 80 µg/kg shellfish meat. This finding underlines clearly that the MBA for lipophilic toxins is neither an appropriate tool for implementing any limit value lower than 160 µg/kg shellfish meat nor is the test capable of providing information on levels close to the regulatory limit, which may be important in the context of the effects of processing.

MBA for STX-group toxins

- The MBA protocol for STX-group toxins is able to quantify these toxins at the current EU regulatory limit value, but not below approximately 370 µg STX equivalents/kg shellfish meat, which is far above the concentration compatible with the ARfD for STX-group toxins.
- The boiling step with hydrochloric acid (HCl) during extraction may result in conversion of less toxic analogues into more toxic ones and thus in an overestimation of the toxicity depending on the toxin profile.

3.1.1.2. Rat bioassay (RBA) for lipophilic toxins

- The RBA is only able to detect toxins that cause diarrhoea. For PTX- and YTX-group toxins, the RBA is not appropriate since those toxins do not cause diarrhoea. For OA-group toxins the detection limit is near 160 µg OA equivalents/kg whilst for AZA-group toxins this has not been established.
- Results of the assay are not quantitative and not objective.

3.1.1.3. High-performance liquid chromatography (HPLC) methods for STX-group toxins and DA

- The HPLC-fluorescence detection (HPLC-FLD) method (Lawrence method) for STX-group toxins is able to quantify at 10-80 µg STX equivalents/kg for individual analogues.
- The HPLC-FLD method (Lawrence method) for STX has not been validated at levels significantly lower than the current EU limit.
- HPLC-based methods are able to quantify DA and epi-domoic acid (epi-DA) concentrations at the current EU limit and also at lower levels e.g. at 4.5 mg DA/kg shellfish meat.

3.1.1.4. Enzyme Linked Immunosorbent Assay (ELISA) method for DA

The ELISA method is able to detect DA and its isomers at the current EU limit and also at lower levels e.g. at 4.5 mg DA/kg shellfish meat.

Table 3a. Performance of official bioassays for the determination of lipophilic marine biotoxins in shellfish as mentioned in EU Regulation (EC) No 2074/2005.

	Toxin group	Ability to perform at the current EU limit	Specificity	Interlaboratory validated/ standardised
Mouse bioassay	OA and analogues	40 % probability to detect at the limit of 160 µg OA eq./kg	None (any lipophilic biotoxin or bioactive compound)	No
	AZA	95 % probability to detect at the limit of 160 µg AZA1 eq./kg	None (any lipophilic biotoxin or bioactive compound)	No
	PTX	Not defined. Appears to be limited chance of detecting 160 µg PTX2 eq./kg	None (any lipophilic biotoxin or bioactive compound)	No
	YTX	Not established High variability	None (any lipophilic biotoxin or bioactive compound)	No
Rat bioassay	OA and analogues	~ 160 µg OA eq./kg	Limited (any lipophilic biotoxin with diarrhoeic effect following oral exposure)	No
	AZA	Not established	Limited (any lipophilic biotoxin with diarrhoeic effect following oral exposure)	No

EU: European Union; eq.: equivalents: OA: okadaic acid; AZA: azaspiracid; PTX: pectenotoxin; YTX: yessotoxin.

Table 3b. Reference methods prescribed in EU legislation for the determination of hydrophilic marine biotoxins, and their LODs and LOQs.

	Toxin group	LOD/LOQ	Specificity	Interlaboratory validated/standardised
Mouse bioassay Regulation (EC) No 2074/2005	STX	LOD: 370 µg STX eq./kg	Limited (any hydrophilic biotoxin with paralytic effects)	Yes AOAC method 959.08
HPLC-FLD (Lawrence method) Regulation (EC) No 1664/2006 amending (EC) No 2074/2005		LOD: not reported LOQ: 10-80 µg STX eq./kg for individual analogues	Adequate, does not separate epimers	Yes AOAC method 2005.06
HPLC-based methods Regulation (EC) No 2074/2005, Regulation (EC) No 1244/2007 amending (EC) No 2074/2005	DA	LOD: 0.2-1 mg DA/kg LOQ: 1-2.5 mg DA/kg	Adequate for DA and epi-DA	Yes AOAC method 991.26 CEN method 14176
Antibody-based methods (ELISA) (For screening purposes) Regulation (EC) No 1244/2007 amending (EC) No 2074/2005		LOD: 0.003 mg DA/kg LOQ: 0.01 mg DA/kg	Adequate for DA	Yes AOAC method 2006.02

EC: European Commission; LOD: limit of detection; LOQ: limit of quantification; LC-FLD: liquid chromatography-fluorescence detection; HPLC: high performance-liquid chromatography; ELISA: Enzyme-Linked Immunosorbent Assay; eq.: equivalents; AOAC: Association of Analytical Communities; CEN: European Committee for Standardization

3.1.2. Alternatives to the reference methods

The alternative methods including their LODs and LOQs, information on their specificity, and information on the interlaboratory validation/standardisation initiatives are presented in Table 4.

For the determination of DA, an improved HPLC-ultraviolet detection (HPLC-UV) analysis procedure was developed by Quilliam *et al*. (1995). This method is sensitive and selective. It has been successfully validated in a collaborative study and standardisation by the European Committee for Standardization (CEN) is currently ongoing. The evidence available suggests that liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) can also be a valuable tool for rapid and selective determination of DA in crude extracts.

For OA-, AZA-, YTX- and PTX-group toxins, the legislation permits replacement of the bioassays provided that the alternative methods have been validated according to an internationally recognised protocol. Currently none of the methods, including the official mammalian bioassays, have been validated by interlaboratory studies. The evidence available at this time suggests that LC-MS/MS based methods have the greatest potential to replace the mammalian assays. These methods can detect the toxins at levels below the current regulatory limit and also have the possibility for multi-toxin group detection/quantification. For OA-group toxins, the phosphoprotein-phosphatase assays, which can also detect the toxins at levels below the current regulatory limit, can also be good candidate alternatives.

Alternative methods for the detection of STX-group toxins involve techniques such as LC-MS/MS, antibody-based sensors and receptor-based assays. None of these methods have yet been interlaboratory-validated according to internationally accepted protocols, so that their performance characteristics cannot be evaluated and compared yet with the official methods. For the antibody-based sensor- and receptor-based methods, such validation studies are in preparation. The biomolecular methods are merely suitable for screening purposes. LC-MS/MS has potential for confirmatory analyses.

The ongoing validation studies listed in Table 4 are designed to control the current EU limits. Recently developed methods (These *et al*, 2009) with lower LODs were successfully tested in prevalidation studies. Method performance criteria should be stipulated where possible and validation by interlaboratory trials should be the long-term objective.

Table 4. Alternative methods for which interlaboratory validation and initiatives for decreasing the LODs are ongoing.

Toxin group	Other methods	Reported LOD (LOQ)	Specificity	Interlaboratory validation/ standardisation initiatives	Recently reported LOD (LOQ)[(a)]
OA and analogues	Phosphoprotein-phosphatase 2a assays - Fluorimetric	26 µg OA eq./kg (41 µg OA eq./kg)	Limited	Interlaboratory study (ES, possibly 2010)	
	Phosphoprotein-phosphatase 2a assays - Colorimetric	10 µg OA eq./kg (32 µg OA eq./kg)	Limited	Interlaboratory study (ES, possibly 2010)	
	LC-FLD	~ 15 µg OA/kg (~ 40 µg OA/kg)	High for individual toxins	Done for OA, not for DTX1 and DTX2 CEN method 14524 (withdrawal recommended by CEN working group)[(b)]	
	LC-MS(/MS)	1-10 µg OA eq./kg (30-50 µg OA eq./kg) (1µg OA eq./kg)[a]	High for individual toxins	Pretrial (NL, 2009) Collaborative trials (DE, 2009; CRL-MB, 2009) Pretrial (BIOTOX project, 2008)	(1µg OA eq./kg)
AZA	LC-MS(/MS)	<1-10 µg AZA1 eq./kg (2-20 µg AZA1 eq./kg) (1 µg AZA1 eq./kg)[a]	High for individual toxins	Pretrial (NL, 2009) Collaborative trial (DE, 2009) Pretrial (BIOTOX project, 2008)	0.3 µg AZA1 eq./kg (1 µg AZA1 eq./kg)
PTX	LC-MS(/MS)	1-4 µg PTX2 eq./kg (1-50 µg PTX2 eq./kg) (1 µg PTX2 eq./kg)[a]	High	Pretrial (BIOTOX project, 2008)	0.3 µg PTX2 eq./kg (1 µg PTX2 eq./kg)

OA: okadaic acid; AZA: azaspiracid; PTX: pectenotoxin; LC-FLD: liquid chromatography-fluorescence detection; LC-MS(/MS): liquid chromatography-mass spectrometry(/mass spectrometry); ELISA: Enzyme-Linked Immunosorbent Assay; eq.: equivalents; CEN: European Committee for Standardization; LOD: limit of detection; LOQ: limit of quantification; NL: The Netherlands; DE: Germany; ES: Spain

(a) Updated LOD/LOQ reported in the literature (These *et al*, 2009)

(b) Resolution 193 of CEN/TC 275/WG-5 Biotoxins (21st Meeting, Paris-France, 16/17 April 2009)

Table 4. Continued

Toxin group	Other methods	Reported LOD (LOQ)	Specificity	Interlaboratory validation/ standardisation initiatives	Recently reported LOD (LOQ)[(a)]
YTX	LC-MS(/MS)	Not reported (0.017 mg/kg shellfish)	High	Collaborative trial (DE, 2009) Pretrial (BIOTOX project, 2008)	> 0.6 µg YTX eq./kg (2 µg YTX eq./kg)
	ELISA	Not reported (0.125 mg YTX eq./kg)	Does not distinguish between different analogues	Interlaboratory study, BIOTOX 2008, publication pending	
STX	Receptor-based assays	Not reported		Interlaboratory study protocol approved (2008), AOAC International	
	Antibody-based methods	Not reported		Prevalidation of sensor-based method (6th FP project BIOCOP, 2009)	
DA	HPLC-UV	0.02-0.03 mg sum DA/kg (Not reported)	DA and epi-DA	Collaborative study CRL-MB, 2003 Standardisation ongoing (CEN)	

YTX: yessotoxin; STX: saxitoxin; DA: domoic acid; LC-MS(/MS): liquid chromatography-mass spectrometry(/mass spectrometry); ELISA: Enzyme-Linked Immunosorbent Assay; HPLC-UV: high performance-liquid chromatography-ultraviolet detection; AOAC: Association of Analytical Communities; CEN: European Committee for Standardization; CRL-MB: Community Reference Laboratory for marine biotoxins; LOD: limit of detection; LOQ: limit of quantification; DE: Germany

(a) Updated LOD/LOQ reported in the literature (These *et al*, 2009)

3.2. Available certified calibrants and reference materials

In all cases analytical methods require reference materials for identification and quantification. Consequently certified reference calibrants for the most frequently occurring analogues and certified tissue reference materials with relevant compositions and levels of toxins should be made available. Commercially available certified calibrants and reference materials are presented in Table 5. The status of certification of the new reference materials can be found on the web sites of the reference material providers: National Research Council Canada- Institute for Marine Biosciences (NRCC-IMB)[16] and Institute for Reference Materials and Measurements (IRMM)[17] .

Table 5. Available certified calibrants and reference materials

Toxin group	List of analogues for which certified reference calibrants are available	Provider	List of certified reference material available	Provider
OA and analogues	OA	NRCC-IMB,	Mussel tissue reference material with OA and DTX1[(a)]	NRCC-IMB
AZA	AZA1	NRCC-IMB,		
YTX	YTX	NRCC-IMB		
STX	N-sulfocarbamoylgonyautoxin-2 and -3 Decarbamoylgonyautoxin-2 and -3 Gonyautoxin-2 and -3 Decarbamoylneosaxitoxin Decarbamoylsaxitoxin Gonyautoxin-1 and -4 Gonyautoxin-5 (B1) Decarbamoylneosaxitoxin Saxitoxin dihydrochloride	NRCC-IMB	Certified lyophilised mussel reference material	IRMM
PTX	PTX2	NRCC-IMB		
DA	DA[(b)]	NRCC-IMB	Mussel tissue reference material with domoic acid[(b)]	NRCC-IMB

CRM: Certified Reference Material; NRCC-IMB: National Research Council Canada- Institute for Marine Biosciences; IRMM: Institute for Reference Materials and Measurements, OA: okadaic acid; AZA: azaspiracid; YTX: yessotoxin; STX Saxitoxin; PTX: pectenotoxin; DA: domoic acid; DTX: dinophysis toxin.
(a): Certified toxin levels are 70-fold higher than current European regulatory limits
(b): The certified value for the calibrant and the mussel tissue reference material relates to the sum of DA and epi-DA.

4. Influence of processing

Based on the limited information available on the effect of processing on levels of lipophilic marine biotoxins in shellfish, the CONTAM Panel concluded that processing of shellfish could lead to an approximate 2-fold increase in the concentration of lipophilic marine biotoxins (OA-, AZA- PTX- and YTX-group toxins) in shellfish meat. Since limit values for marine biotoxins in shellfish meat are meant to protect the consumer, the effect of processing (cooking, steaming, autoclaving) should be considered when testing shellfish in official control. Shellfish that contains levels of lipophilic toxins

[16] www. nrc-cnrc.gc.ca
[17] http://irmm.jrc.ec.europa.eu

below the regulatory limits may reach, after processing, levels that are higher than the regulatory limits. For OA-, AZA- and PTX-group toxins, the MBA is not quantitative and is not capable of detecting concentrations below their current EU regulatory limit. Thus, effects of commercial processing cannot be monitored using the MBA.

Concerning hydrophilic marine biotoxins, water loss during household processing (cooking, steaming) of shellfish could lead to leaching-out of STX-group toxins from the flesh into the cooking fluid. The CONTAM Panel concluded, however, that the available information made it difficult to draw firm conclusions on possible interconversion or destruction of STX-group toxins occurring during commercial processing.

The effects of cooking on the concentration of DA and epi-DA in shellfish vary between species. In scallops redistribution of the toxins during cooking and leaching out of the toxins into the cooking fluid may lead to a reduction of the concentration of DA and epi-DA in the hepatopancreas and to an increase of the concentration in the whole body excluding the hepatopancreas. For other types of shellfish it is unlikely that processing would have a major effect on the DA and epi-DA concentration in shellfish meat.

5. Relative potency of analogues

TEFs have been used to convert the concentrations of the OA-, AZA-, YTX-, STX- and PTX-group toxins respectively into OA, AZA1, YTX, STX and PTX2 equivalents in order to allow for the combined toxicity of the different analogues. The TEF values adopted by the CONTAM Panel, based on acute toxicity following *i.p.* administration to mice, are presented in Table 6.

The limited toxicological information does not allow the setting of robust TEFs for the oral route for any of the toxin groups. Even for the *i.p* route, the available toxicity data are very limited for the AZA-, YTX- and PTX-group toxins. Further toxicological data are needed for the establishment of robust TEFs for the oral route of administration for all toxin groups. The assumption of dose additivity should be assessed following exposure to combinations of toxin analogues and milligram amounts of purified toxins should be produced for this purpose. The TEF values should be revised when studies on acute oral toxicity data for the relevant analogues of each toxin group become available.

Table 6. TEFs adopted by the CONTAM Panel for regulated marine biotoxins.

Toxin group	Analogue	TEF
OA-group toxins (OA-equivalents)	OA	1
	DTX1	1
	DTX2	0.6
AZA-group toxins (AZA-equivalents)	AZA1	1
	AZA2	1.8
	AZA3	1.4
YTX-group toxins (YTX-equivalents)	YTX	1
	1a-homoYTX	1
	45-hydroxyYTX	1
	45-hydroxy-1a-homoYTX	0.5
STX-group toxins (STX-equivalents)	STX	1
	NeoSTX	1
	GTX1	1
	GTX2	0.4
	GTX3	0.6
	GTX4	0.7
	GTX5	0.1
	GTX6	0.1
	C2	0.1
	C4	0.1
	dc-STX = 1	1
	dc-NeoSTX	0.4
	dc GTX2	0.2
	dc GTX3	0.4
PTX-group toxins (PTX2-equivalents)	PTX1	1
	PTX2	1
	PTX3	1
	PTX4	1
	PTX6	1
	PTX11	1
DA and its isomers	None established	-

TEF: Toxicity equivalency factors; OA: okadaic acid; AZA: azaspiracid; YTX: yessotoxin; STX: saxitoxin; PTX: pectenotoxin; DA; domoic acid; DTX: dinophysis toxin; GTX: gonyautoxin; dcGTX: decarbamoyl gonyautoxin; dcNeoSTX: decarbamoyl neosaxitoxin; dcSTX: decarbamoyl saxitoxin.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

- The Scientific Panel on Contaminants in the Food Chain has established acute reference doses for the currently regulated marine biotoxins. These are amounts of the toxins, expressed on a body weight basis, that can be consumed within 24 hours or less without appreciable risk to health.
- From the available consumption data for shellfish, 400 g is identified as a realistic estimate of a large portion size, which should be used in the risk assessment in order to protect high consumers from the acute effects of the marine biotoxins.
- Based on the currently available data it appears that the current European Union (EU) regulatory limit values for okadaic acid-group, azapiracid-group, saxitoxin (STX)-group toxins and domoic acid (DA) are not sufficiently protective for consumers.

- For yessotoxin- and pectenotoxin-group toxins, the EU limit values appear to be sufficiently protective for consumers.
- The mouse bioassay (MBA) for lipophilic biotoxins has shortcomings and is not considered an appropriate tool for control purposes because of the high variability in results, the insufficient detection capability and the limited specificity.
- The MBA is not capable of detecting concentrations considerably below the current EU levels. Thus, effects of commercial processing on lipophilic biotoxins cannot be monitored using the MBA. Since limit values for marine biotoxins in shellfish meat are meant to protect the consumer, the effect of processing should be considered when testing shellfish in official control.
- For lipophilic biotoxins the multitoxin-methods based on liquid chromatography-mass spectrometry/mass spectrometry are specific, have sufficient limits of detection and therefore the greatest potential to replace the mammalian bioassays.
- For hydrophilic biotoxins the MBA is able to quantify STX-group toxins at the current EU regulatory limit, but not below approximately 370 µg STX equivalents/kg shellfish meat. The boiling step with hydrochloric acid during extraction may result in overestimation of the toxicity depending on the toxin profile, because of transformation of less toxic analogues into more toxic ones.
- The high performance liquid chromatography (HPLC)-fluorescence detection method (Lawrence method) for STX-group toxins is able to quantify 10-80 µg STX equivalents/kg for individual analogues, but has not been validated for lower levels.
- HPLC-based methods are able to quantify DA and epi-domoic acid concentrations at the current EU limit and also at the lower levels e.g. 4.5 mg DA/kg shellfish meat.

RECOMMENDATIONS (INCL. KNOWLEDGE/DATA GAPS)

- Reporting systems for human illness associated with marine biotoxins should be improved to better reflect the incidences and to allow for assessment of toxin exposure.
- The database on shellfish consumption should be extended, including portion size and frequency of consumption for different types of shellfish.
- Further data on the effects of processing on levels of marine biotoxins in shellfish are needed.
- Toxicity equivalency factors should be established on the basis of acute oral toxicity data for toxin analogues that are toxicologically relevant at the levels occurring in shellfish.
- Additional information on genotoxicity, oral toxicity and mechanisms of toxicity is required for some toxin groups.
- Information is needed on the combined toxicity of different toxin groups that often co-occur in contaminated shellfish.
- Further intensified efforts are needed for formal interlaboratory validation of methods.
- Further developments of functional and biomolecular methods for marine biotoxin detection, as well as better characterisation of their performance characteristics are needed.
- Certified reference calibrants/materials, at least for the regulated analogues, are required to reliably quantify these analogues in shellfish to make enforcement of regulations possible, and to evaluate the risk posed by their occurrence.

- Performance criteria should be established for analytical methods to be used for the determination of marine biotoxins for official control purposes.

REFERENCES

Hess P, Butter T, Peterson A, Silke J and McMahon T, 2009. Performance of the EU-harmonised mouse bioassay for lipophilic toxins for the detection of azaspiracids in naturally contaminated mussel (*Mytilus edulis*) hepatopancreas tissue homogenates characterised by liquid chromatography coupled to tandem mass spectrometry. *Toxicon* 53,713-722.

Quilliam MA, Xie M and Hardstaff WR, 1995. Rapid extraction and cleanup for liquid-chromatographic determination of domoic acid in unsalted seafood. *Journal of AOAC International* 78 (2), 543-554.

These A, Scholz J and Preiss-Wiegert A, 2009. Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography–tandem mass spectrometry based on enrichment by solid-phase extraction. *Journal of Chromatography* A 1216 (21), 4529-4538.

ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
ARfD	Acute reference dose
AZA	Azaspiracid
b.w.	Body weight
CEN	European Committee for Standardization
CONTAM Panel	Panel on Contaminants in the Food chain
CRL-MB	Community Reference Laboratory for Marine Biotoxins
CRM	Certified Reference Material
dcGTX	Decarbamoyl gonyautoxin
dcNeoSTX	Decarbamoyl neosaxitoxin
dcSTX	Decarbamoyl saxitoxin
DA	Domoic acid
DE	Germany
DTX	Dinophysis toxins
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Aassay
eq.	Equivalent
ES	Spain
EU	European Union
GTX	Gonyautoxin
HPLC	High-Performance Liquid Chromatography
HPLC-FLD	High-Performance Liquid-Fluorescence Detection
HPLC-UV	High-Performance Liquid Chromatography - Ultraviolet detection
i.p	Intraperitoneal
IRMM	Institute for Reference Materials and Measurements
LB	Lower Bound
LC-MS(/MS)	Liquid chromatography- mass spectrometry (/mass spectrometry)
LOD	Limit of detection
LOQ	Limit of quantification
MBA	Mouse bioassay
NL	The Netherlands
NRCC-IMB	National Research Council Canada - Institute for Marine Biosciences
OA	Okadaic acid
PTX	Pectenotoxin
PSP	Paralytic Shellfish Poison

RBA	Rat bioassay
SM	Shellfish meat
STX	Saxitoxin
TEF	Toxicity equivalency factor
UB	Upper Bound
YTX	Yessotoxin